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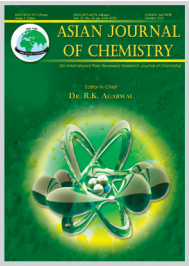

Structural Elucidation of A Bioactive Compound from the Leaves of *Neptunia prostrate*

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The analgesic activity of the methanolic extract of leaves of *Neptunia prostrate* and its separated fractions were studied in acetic acid induced model. The *in vivo* antiinflammatory was also studied using Carageenan induced rat paw edema. The result of both the studies indicated that the extract and its components possessed significant analgesic activity as well as antiinflammatory activity at the dose 100 mg/Kg. Both the activities were compared with standard drug aspirin for analgesic and diclofenance sodium for antiinflammatory. The structure of the bioactive compound has been characterized on the basis of spectral data such as IR, ^{1}H NMR, ^{13}C NMR and mass spectral studies.

Key Words: *Neptunia prostrate*, Analgesic activity, Antiinflammatory activity.

INTRODUCTION

It is believed that current analgesic and antiinflammatory drugs such as opiodes and NSAID are not useful in all cases. The prolonged use of these drugs may lead to serious side-effects such as gastric intolerance, bone marrow depression, water and salt retention. For this reason, there is a need to find and develop new antiinflammatory and analgesic drugs with low side-effect. Naturally originated new agents with little side-effects can replace chemical pharmaceuticals[1]. Inflammation is a normal protective response to tissue injury caused by physical trauma, noxious chemical or microbial agents[2,3]. Modern research in the field of antiinflammatory drugs is directed towards developing potent antiinflammatory compounds with improved tolerability and reduction in other major side effects. Drugs from plant origin are used in India for treatment of many diseases in traditional system of medicine[4]. Indigenous drug system or Ayurveda can be source of variety of new drugs, which can provide relief from inflammation, but their claimed reputation has to be verified on a scientific basis. In some cases indigenous drugs may be the only answer because these drugs have minimum side effects and are easily available at low cost[5].

The objective of this study is to determine the analgesic activity and antiinflammatory activity of the leaves of *Neptunia prostrate* to compare with those of conventional antiinflammatory and antinociceptive drugs. *Neptunia prostrate* (Mimosaceae) is an aquatic herb. In this study, we concentrate on the analgesic and antiinflammatory activity and ultimately the structural elucidation of the bioactive compound from the leaves of *Neptunia prostrate*. This plant is used as vegetable. Tribal cook it as sag. They remove the spongy tissues of the stem and take the inner soft tender portion along with leaves. Leaves are used as condiments[6].

It is distributed in south east Asia, Malaysia and Argentina[7-9]. In the present study, the evaluation of analgesic and antiinflammatory effects were undertaken using acetic acid- induced writhing test and carrageenan-induced paw edema test. Traditionally it is used in acidity, gastritis and constipation[10].

EXPERIMENTAL

Sample preparation: The leaves of *Neptunia prostrata* L. were taken, which were collected from Agartala in the month of May 2009 and were shed dried for seven days. Since certain compounds get denatured in sunlight, it is dried under shade to avoid decomposition. The dried leaves were then crushed to fine powder. The powdered content was extracted by methanol in a Soxhlet apparatus by taking 100 g powder in 100 mL of methanol.

Separation: Components present in the extract were separated by following column chromatography technique taking ethyl acetate as mobile phase and silica gel, as stationary phase. The column used was of 30 cm in length. Separated fractions were collected in individual beakers. The colour of the components and volume obtained is given in Table-1.

TABLE–1
COLOUR OF THE COMPONENTS AND VOLUME OBTAINED AFTER SEPARATION BY COLUMN CHROMATOGRAPHY

Name of the plant	Components separated (eluted)	Colour	Volume collected (mL)
Neptunia prostrate	NP_1 (1^{st})	Green	19.0
	NP_2 (2^{nd})	Brown	1.0
	NP_3 (3^{rd})	Deep green	15.5
	NP_4 (4^{th})	Brownish green	12.0
	Absorbed	-	2.5

Melting point of all compounds were determined in open capillaries by melting point apparatus (INDO, M-AB-92) and are uncorrected. The IR spectra were recorded (in Affinity-1 FTIR spectrophotometer IR solution Version 1.50 SU Shimadzu corporation) in KBr pellets. ^1H, ^{13}C-NMR were run on Mercury-400BB in $CDCl_3$, while mass spectra was performed in Agilent 7890 GC coupled with 5975 MS.

Screening of analgesic activity (acetic acid induced writhing in mice)[11]: Albino mice of either sex (weighing 25-30 g) were used as per experimental protocols approved by Institute of Bioresources and sustainable development (IBSD), Department of Biotechnology, Government of India, Imphal, Manipur. The animals were housed under standard environmental condition (25 ± 2 ºC) and relative humidity (50 ± 5 %) and fed with standard diet and water adlibitum. The animals were acclimatized to laboratory environment for a period of 14 days before performing the experiments.

The mice were divided into five groups of five animals each. The first group comprised the control and the remaining four groups were administered test dose. The test doses were prepared in distilled water to get the desired concentration of the extract and the separated compounds.

Acetic acid (1 % v/v, 10 mL/kg) was injected into the peritoneal cavities of mice, which were placed in a large plastic tray and the intensity of nociceptive behaviour was quantified by the number of writhes which was counted for 10 min beginning from 5 min after the acetic acid injection. Test drugs and control vehicle were administered 0.5 h before the acetic acid injection. The writhing response consists of a contraction of the abdominal muscle turning of trunk (twist) together with a stretching of the hind limbs. The antinoceptive activity was expressed as the writhing scores over 20 min. Percent inhibition of writhing was calculated using the relation:

$$\text{Inhibition of writhing (\%)} = 100\left(1 - \frac{\text{Mean writhing number of treated mice}}{\text{Mean writhing number of control mice}}\right)$$

Results are depicted in Table- 2.

Anti-inflammatory activity[12]: Antiinflammatory activity was evaluated using 0.1 mL of carrageenan (1 % w/v) induced hind paw edema method. Albino rats of either sex between 150-200 g were selected for the studies. The animals were kept on diet and allowed food and water *ad libitum*. They were housed in polypropylene cages maintained under condition (12 h light/12 h. dark cycle ± 3 ºC, 35 -60 % humidity). The rats were divided into four groups of five rats each. The test groups-3 and 4 received 200 mg/Kg of compound NP_2 and extract NP_E by oral route. The positive control received (2^{nd} group) standard diclofenace sodium (8 mg/Kg P.O.) by oral route. All the suspensions were administered 1 h before the injection of Carrageenan (0.1 mL of 1 % w/v). The hind paw volume was measured plethysmometrically before and after the carrageenam injection at hourly intervals for 5 h and percentage inhibition of inflammation was calculated using the relation:

$$\text{Inhibition of oedema (\%)} = 100\left(1 - \frac{\text{Mean paw volume of treated rats}}{\text{Mean paw volume of control rats}}\right)$$

Results are displayed in Tables 3 and 4.

RESULTS AND DISCUSSION

The methanolic extract (200 mg/Kg) suppressed the acetic acid induced writhing response significantly. The results were found to be highly significant ($P < 0.001$) in comparison to the control. Sample NP_2 showed better percentage of protection as compared to other samples. Aspirin, which has average writhing of 3.8 ± 0.387, is a synthetic compared. So comparing this with that of sample NP_2, average writhing of 4.2 ± 0.7348, a natural product is showing a much better analgesic activity.

Both the sample showed better antiinflammatory activity when compared to control. Sample NP_2 and NP_E showed significant result after 3 h. But the % protection was found to be better for sample NP_2 compared to sample NP_E. Significant %

TABLE-2
RESULTS OF ANALGESIC ACTIVITY (ACETIC ACID–INDUCES WRITHING)

Animal no.	Treatment	Dose	Number of writhing (10 min duration)	Responders (n/n)	Mean of writhing ± standard error mean (SEM)	Inhibition (%)
1			14			
2	Control Water	10 mL/kg	12			
3	+	+	15	5/5	14.8 ± 0.8602	0
4	(Acetic acid)	0.1 mL/kg	16			
5			17			
1			03			
2	Sample NP_2	200 mg/kg	05			
3	+	+	02	5/5	4.2 ± 0.7348	71.7
4	(Acetic acid)	0.1 mL/kg	05			
5			06			
1			07			
2	Sample NP_E	200 mg/kg	05			
3	+	+	04	5/5	5.0 ± 0.5477	66.3
4	(Acetic acid)	0.1 mL/kg	05			
5			04			

TABLE-3
EFFECT OF UNKNOWN SAMPLES ON CARRAGEENIN INDUCED PAW EDEMA IN RATS

Treatment	Paw volume (mL)				
	0 h	1 h	2 h	3 h	5 h
Normal control (1 mL dist. water P.O.)	0.30	0.40	0.60	0.65	0.65
	0.30	0.45	0.55	0.55	0.50
	0.20	0.30	0.45	0.55	0.50
	0.20	0.35	0.60	0.75	0.70
	0.20	0.30	0.55	0.65	0.60
Standard diclofenace sodium (8 mg/kg P.O.)	0.20	0.35	0.45	0.40	0.30
	0.20	0.30	0.40	0.40	0.35
	0.25	0.35	0.40	0.35	0.25
	0.30	0.40	0.45	0.40	0.30
	0.35	0.45	0.35	0.45	0.40
Sample NP_2	0.35	0.40	0.45	0.40	0.30
	0.25	0.30	0.35	0.35	0.30
	0.30	0.40	0.50	0.45	0.40
	0.20	0.25	0.30	0.30	0.25
	0.25	0.35	0.40	0.35	0.30
Sample NP_E	0.25	0.30	0.35	0.30	0.30
	0.20	0.25	0.30	0.25	0.25
	0.35	0.45	0.50	0.45	0.40
	0.30	0.35	0.40	0.35	0.30
	0.25	0.35	0.40	0.40	0.35

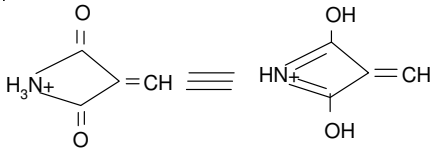
of protection was found for both the samples NP_2 and NP_E continued after 4 h just as it was found for diclofenace sodium.

The melting point of the NP_2 compound was found to be 121 ºC. In the IR spectral studies in KBr pelletes, the peak at 3400 cm^{-1} can be assigned to H-N -1º (aromatic) stretching. The peak at 2924 cm^{-1} can be correlated to CH=CH stretching. The peak at 2853 cm^{-1} is due to C-H stretching. The peak at 1715 cm^{-1} is due to α,β-unsaturated C=O stretching conjugated with aromatic ring. The peak at 1652 cm^{-1} is due to C=C stretching, The peak at 1384 cm^{-1} $(CH_3)_3$-C stretching 1384 cm^{-1}. The peak 1179 cm^{-1} is due to =C-C=C-C stretching.

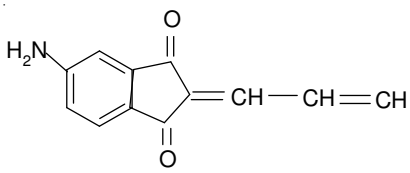
In the ^{1}H NMR spectral analysis signal near 3.29 (singlet) is for 1º-$ArNH_2$, peak at 7.25 (singlet) is for aromatic protons, peak at 2.63 (duplet) is for ^{1}CH near to cyclopanta-none, peaks at 2.15, 2.23, 2.27 (multiplet) is for ^{2}CH and ^{3}CH, peak at 2.02 (duplet) is for CH_2, peak at 1.35 (singlet) is for $^3CH_3$.

In the ^{13}C NMR spectra, the peak around 199.053 is due to ^{5}C and ^{7}C, the peak around 182.679 is due to ^{1}C. The peak around 78.183 is due to ^{8}C and ^{4}C, the peaks around 77.542, 77.230, 76.909 is due to ^{2}C, ^{9}C and ^{3}C, the peaks around 37.649, 32.135 are due to ^{6}C and ^{10}C respectively, the peaks around 29.908, 26.202, 24.730, 22.899 are due to ^{11}C, ^{12}C, ^{13}C and ^{14}C respectively, the peaks around 19.521, 14.312 is due to ^{15}C, ^{16}C and ^{17}C.

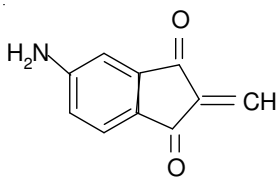
In mass spectral analysis the compound showed a molecular ion peak $(M+1)^+$ at m/z 270. The base peak was recorded as m/z 74 for $C_6H_2^+$, as NH_2 may take away one adjacent H and diketone part may be in the form (m/z 98)-



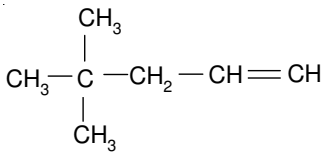
The other fragments $(m/z)^+$ obtained may be described as under-



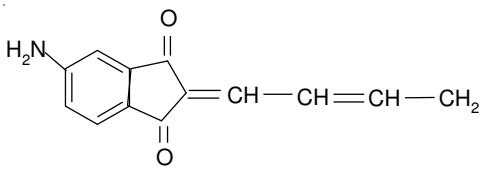
M+1=198



M+1=172

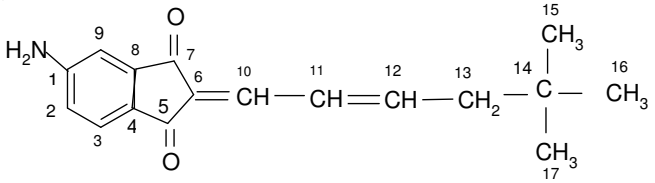


M = 97



M+1=212

Based on the above spectral analysis the structure may be drawn as-

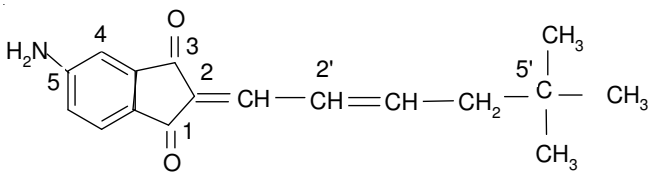


Conclusion

Sample NP_2, a natural product, shows a better analgesic activity and antiinflammatory activity. Based on the above spectral analysis the structure may be drawn as-

TABLE-4
PROTECTION OF THE UNKNOWN SAMPLE (%)

Treatment	Mean ± SEM of paw volume (mL)					
	0 h	1 h	2 h	3 h	5 h	Protection (%)
Normal control (1 mL dist. water P.O.)	0.24 ± 0.02449	0.36 ± 0.02915	0.55 ± 0.02739	0.63 ± 0.03742	0.59 ± 0.04000	0
Standard diclofenace sodium (8 mg/kg P.O.)	0.26 ± 0.02915	0.37 ± 0.02550	0.44 ± 0.01871	0.40 ± 0.01581	0.32 ± 0.02550	45.7
Sample NP_2	0.27 ± 0.02550	0.37 ± 0.02550	0.40 ± 0.03536	0.37 ± 0.02550	0.31 ± 0.02449	47.45
Sample NP_E	0.27 ± 0.02550	0.34 ± 0.03317	0.39 ± 0.03317	0.35 ± 0.03536	0.32 ± 0.02550	45.7



The compound can be named as 5-amino-2-(5',5'-dimethylhex-2'-en-1'-ylidene)-indene-1,3-dione.

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